Regulation of Anoikis

<Review>

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ABSTRACT

Normal epithelial and endothelial cells require attachment to extracellular matrix (ECM) proteins to grow or survive. In these anchorage-dependent cells, loss of interaction with the ECM proteins triggers apoptosis which is termed anoikis. Anoikis undoubtedly plays an essential role in the development and organization of normal tissues through its inhibitory effect on unfavorable cellular proliferation at inappropriate locations. In this regard, anoikis contributes to the maintenance of the physiological state. Importantly, disturbance of anoikis may allow cell proliferation at inappropriate sites and thus may be tightly linked to cancer development. Indeed, we have found that suppression of anoikis promotes peritoneal dissemination or metastasis of several carcinoma cells. These data imply that clarification of the molecular mechanism which regulates anoikis will, in turn, greatly help the regulation of cancer progression. Here we summarize recent advances in the field of anoikis regulation.

Key words : Anoikis, Apoptosis, Akt, Bcl-2, BH3-only proteins, Integrin

1. Introduction

Epithelial, endothelial and muscle cells require tight interaction with extracellular matrix (ECM) proteins to grow or survive. When these anchorage-dependent cells lose this interaction, they gradually fall into apoptosis, which is termed anoikis[1]. Anoikis is considered to play a pivotal role in the development and organization of normal tissues through its inhibitory effect on unfavorable cellular proliferation at inappropriate locations. This implies that disturbance of anoikis may allow inappropriate cell growth and that it is thus tightly linked to cancer development. In this regard, regulation of anoikis may be crucial for an understanding of cancer and other abnormal developments.

Normal epithelial cells attach to the ECM proteins through integrin receptors. Thus, loss of anchorage leads to inactivation of integrin receptor-mediated signals in anchorage-dependent cells. To date, it has been revealed that lots of signaling pathways link to integrin receptors and protect epithelial cells from anoikis. Ligand-bound integrin receptors activate both focal adhesion kinase, FAK[2] and integrin-linked kinase, ILK[3], which introduce tyrosine and serine/

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threonine phosphorylation, respectively. Importantly, FAK modulates the organization of cytoskeletal proteins, while ILK strongly activates PKB/Akt[4]. These kinases introduce cell survival signals, and thus play central roles in suppression of anoikis. Conversely, phosphatases may reverse their inhibitory effects on anoikis. In this regard, phosphorylation is a central event following integrin receptor-mediated signals. Additionally, it is also thought that apoptosis-related molecules, Bcl-2 family members, caspases and their regulators, may contribute to the regulation of anoikis either dependently or independently of phosphorylation events. Since the functions of these molecules are not directly linked to cell attachment, their contribution remains uncertain. It seems rather likely that phosphorylation events play central roles in the regulation of anoikis. Among the integrin receptor-coupled kinases, Akt seems to play a pivotal role in the suppression of anoikis, since a variety of molecules implicated in the apoptotic process have been identified as substrates of Akt. In this paper, we focus on phosphorylation events and describe recent advances concerning regulation of anoikis.

2. Positive regulators of anoikis

In anchorage-dependent cells, loss of anchorage leads to several apoptotic signals as shown in Fig. 1. It has been found that Jun N-terminal kinase (JNK) is activated in and promotes anoikis in a manner which is dependent on increased caspase activity[5]. Namely, loss of anchorage activates DEVD motif-specific caspases, which can cleave MEKK-1 specifically to remove an inhibitory N-terminal domain, thereafter activating JNK as a downstream event[6]. However, the results of one report controversially showed that JNK activation is not required for anoikis to proceed[7]. Thus, JNK activation may not be an initial event, but rather a secondary event following anoikis.

Caspase-8 is also activated during anoikis[8, 9]. Caspase-8 is a member of the apoptosis operator caspases, and is activated by oligomerization with FAS-associated death domain protein (FADD). Both caspase-8 activation and subsequent cell death from anoikis are strongly inhibited by dominant-negative FADD, however inhibition of CD95, DR4 and DR5 receptors fails to block the events. In addition, the events occur at an initial stage of anoikis. Thus, FADD-mediated caspase-8 activation is a crucial event for anoikis to proceed. However, the precise molecular mechanism by which FADD-mediated caspase-8 activation takes place remains to be clarified.

The possible involvement of PTEN in the regulation of anoikis is suggested[10]. PTEN (phosphatase and tensin homolog deleted from chromosome 10) is a tumor suppressor gene, which suffers loss of heterozygosity in many human cancers and somatic mutations or deletions in a large fraction of tumors. PTEN contains a protein tyrosine phosphatase domain and negatively regulates FAK and Shc. Importantly, it has been reported that reintroduction of PTEN into the cells carrying mutated PTEN leads to direct dephosphorylation of FAK by PTEN, thereby inhibiting the integrin-mediated cell survival signal. In addition, recent study has revealed that PTEN also inhibits ILK-mediated Akt activation in prostate cancer cells[11]. From these data, PTEN is believed to be a positive regulator of anoikis by inactivation of FAK and ILK.

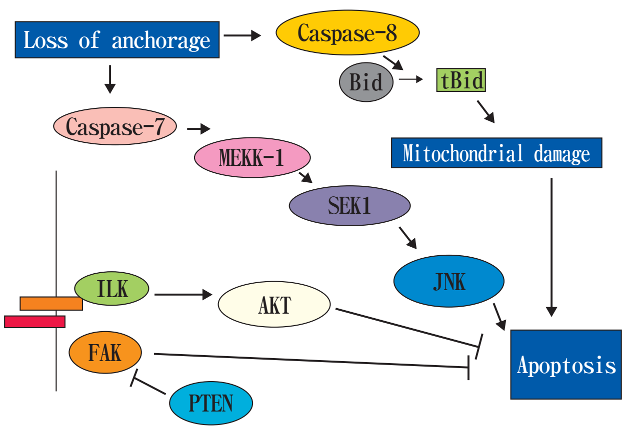

Fig. 1 Positive regulators of anoikis

3. Negative regulators of anoikis

As described above, integrin-mediated signals protect epithelial cells from anoikis and are considered to have a central role in negative regulation of anoikis (Fig. 2). Akt is the best candidate for negative regulator of anoikis since a variety of molecules implicated in the apoptotic process, such as the Bcl-2 family member BAD[12], IkB kinase[13] and caspase-9[14], have been identified as substrates of Akt. BAD is a BH3-only protein and functions as a proapoptotic molecule. Importantly, BAD is negatively regulated by Akt through direct phosphorylation at three serine residues[12]. Akt also phosphorylates IkB and activates its enzymatic activity[13]. Since activated IkB kinase negates the interaction of IkB with NF-kB by direct phosphorylation, leading to activation of NF-kB-mediated signals, Akt eventually activates NF-kB signals, which contribute as strong survival signals in a variety of cell types. Phosphorylation of caspase-9 leads to its rapid degradation and the termination of caspase-dependent apoptotic signals[14].

Integrin-mediated signals also introduce tyrosine phosphorylation events through activation of FAK. Constitutively activated forms of FAK rescued at least two established epithelial

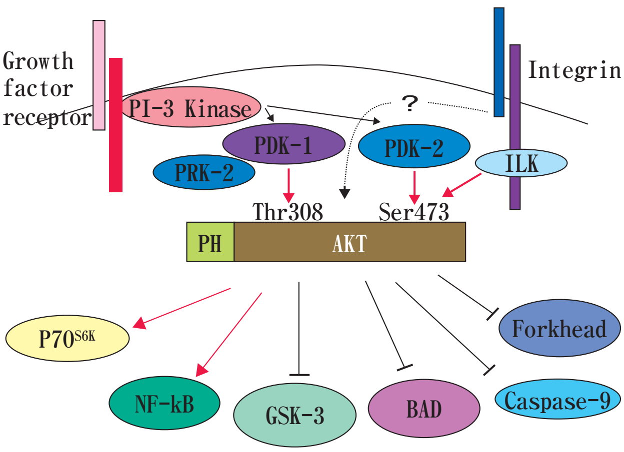

Fig. 2 Integrin receptor-mediated signals

cell lines from anoikis[15]. Importantly, activated FAK can transform MDCK cells, by the criteria of anchorage-independence and tumor formation in nude mice. Thus, the data strongly suggest that this type of transformation results primarily from resistance to anoikis rather than from aberrant cell proliferation, implying a crucial role for anoikis in oncogenesis. To date, it has been found that FAK interacts with a number of cellular proteins, including c-src, GRB2, phosphatidylinositol-3-kinase, paxillin, and $p130^{cas}$. Although these signaling molecules may contribute to FAK-mediated suppression of anoikis, current studies, which have failed to identify the molecule directly responsible for protecting cells from anoikis, that suggest their contribution is unlikely. Thus, it remains to be elucidated how FAK prevents anoikis.

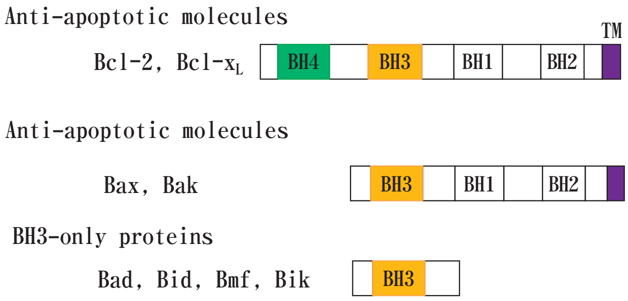

Fig. 3 Structures of Bcl-2 family members

4. BH3-only proteins BAD and Bmf

BAD is a BH3-only protein among the Bcl-2 family members (Fig. 3). BAD is ubiquitously expressed in a variety of normal tissues, and at its highest level in epithelial cells[16]. Thus, BAD may contribute to cell death preferentially in epithelial cells. As described above, normal epithelial cells are anchorage-dependent, and their life seems to be maintained mainly by the interaction of integrin receptors with ECM proteins. Since BAD is abundantly expressed in these cells, and is negatively regulated by Akt, BAD is likely to be a main regulator of their life.

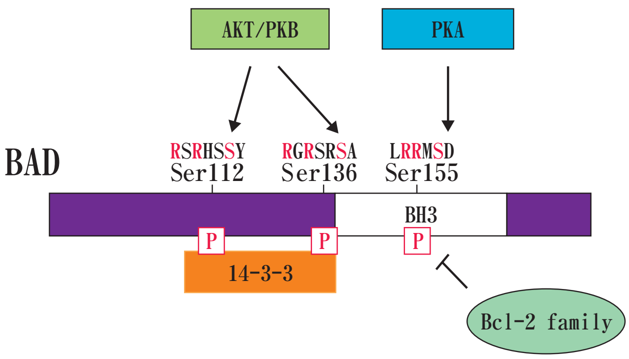

Fig. 4 Phosphorylation sites of BAD

Although BAD is activated by an increase in its protein levels, the activity is tightly regulated by its phosphorylation levels. These are at least three major phosphorylation sites in the BAD molecule (Fig. 4). The $p90^{RSK}$ or PKB/Akt phosphorylates BAD at Ser112/Ser136[12, 17] (28, 31). PKA also phosphorylates BAD at Ser155 which inactivates the pro-apoptotic function of BAD[18]. Highly phosphorylated BAD resides in the cytoplasm as an inactive form and preferentially binds to 14-3-3[19, 20]. Ligand-bound integrin receptors introduce signals to maintain BAD in a highly phosphorylated state by activated Akt. Conversely, dephosphorylation of BAD leads to loss of ability to interact with 14-3-3, and thereafter interaction takes place with anti-apoptotic Bcl-2 family members Bcl-X_L and Bcl-2 at the mitochondrial surface[21-23]. Recently, we clearly demonstrate that BAD selectively augments anoikis in MDCK cells. In addition, this effect is totally abrogated by co-transfection of constitutively active Akt (to be published elswere). These data strongly suggest that BAD is a positive regulator of anoikis.

In addition, proapoptotic Bcl-2 protein BAX is suggested to be involved in anoikis[24]. As described above, caspase-8 is activated by anoikis. Importantly, activated caspase-8 can generate a cleaved form of Bid (tBid)[25], which preferentially interact with BAX. The tBid-BAX interaction results in a conformational change of BAX, and leads to exposure of the two central helices and the carboxy-terminal transmembrane domain to facilitate mitochondrial membrane insertion, oligomerization, and pore formation. In this regard, BAX may be an operator of caspase-8-mediated cell death, and thus the involvement of BAX in anoikis may not be a specific event.

More recently, another proapoptotic BH3-only protein, Bmf, has been identified. Bmf is sequestered to myosin V motors by association with dynein light chain 2[26]. Loss of cell attachment unleash Bmf, allowing it to translocate and bind antiapoptotic Bcl-2 proteins. This strongly suggests that Bmf is a new molecule which can detect intracellular damage in cytoskeletal structures and introduce apoptotic signals. In this regard, Bmf is a good positive regulator of anoikis.

5. Conclusions

In this paper, we describe recent advances in the understanding of anoikis regulation. Anoikis is obviously an important event not only in cancer progression but also in organ development. We have already shown that antiapoptosis promotes metastasis and dissemination of carcinoma cells[27,28]. In addition, we have also developed the adenovirus-mediated caspase-8 expression system and demonstrated that this system can reduce peritoneal dissemination of gastric cancer cells[29]. The data encourage us to try to develop a novel anti-metastatic therapy based on the augmentation of anoikis. In addition, we emphasize that regulation of anoikis is crucial for reconstitution of organs, since unfavorable cell growth must be avoided if normal tissue formation is to occur. In this regard, clarification of the process of anoikis and its regulation is perhaps the most attracting issue in the field of medical science.

REFERENCES

1. Frisch SM, Francis HJ. Disruption of epithelial cell matrix interactions induces apoptosis. J Cell Biol 1994, 124: 619–626.
2. Ruoslahti E, Reed JC. Anchorage dependence, integrins, and apoptosis. Cell 1994, 77: 477–478.
3. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. Oncogene 2000, 19: 3811–3815.
4. Khwaja AP, Rodriguez-Viciana S, Wennstrom P, Warne H, Downward J. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 1997, 16: 2783–2793.
5. Frisch SM, Vuori K, Kelaita D, Sicks S. A role for Jun-N-terminal kinase in anoikis; suppression by bcl-2 and crmA. J Cell Biol 1996, 135: 1377–1382.

6. Cardone MH, Salvesen GS, Widmann C, Johnson G, Frisch SM. The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. Cell 1997, 90: 315-323.
7. Khwaja A, Downward J. Lack of correlation between activation of Jun-NH2-terminal kinase and induction of apoptosis after detachment of epithelial cells. J Cell Biol 1997, 139: 1017-1023.
8. Rytomaa M, Martins LM, Downward J. Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. Curr Biol 1999, 9: 1043-1046.
9. Frisch SM. Evidence for a finction of death-receptor-related, death-domain-containing proteins in anoikis. Curr Biol 1999, 9: 1047-1049.
10. Cristofano AD, Pandolfi PP. The multiple roles of PTEN in tumor suppression. Cell 2000, 100: 387-390.
11. Persad S, Attwell S, Gray V, Delcommenne M, Troussard A, Sanghera J, Dedhar S. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. Proc Natl Acad Sci USA 2000, 97: 3207-3217.
12. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997, 91: 231-241.
13. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999, 401: 86-90.
14. Cardone MH., Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998, 282: 1318-1321.
15. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol 1996, 134: 793-799.
16. Kitada S, Krajewska M, Zhang X, Scudiero D, Zapata JM, Wang, HG, Shabaik A, Tudor G, Krajewski S, Myers TG, Johnson GS, Sausville EA, Reed JC. Expression and location of pro-apoptotic Bcl-2 family protein BAD in normal human tissues and tumor cell lines. Am J Pathol 1998, 152: 51-61.
17. Tan Y, Ruan H, Demeter MR, Comb MJ. p 90(RSK) blocks bad-mediated cell death via a protein kinase C-dependent pathway. J Biol Chem 1999, 274: 34859-34867.
18. Tan Y, Demeter MR, Ruan H, Comb MJ. BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. J Biol Chem 2000, 275: 25865-25869.
19. Zhou XM, Liu Y, Payne G, Lutz RJ, Chittenden T. Growth factors inactivate the cell death promoter BAD by phosphorylation of its BH3 domain on Ser155. J Biol Chem 2000, 275: 25046-25051.
20. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-XL. Cell 1996, 87: 619-628.
21. Ayllon V, Martinez-A C, Garcia A, Cayla X, Rebollo A. Protein phosphatase 1α is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. EMBO J 2000, 19: 2237-2246.
22. Chiang CW, Harris G, Ellig C, Masters SC, Subramanian R, Shenolikar S, Wadzinski BE, Yang E. Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin- 3-dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation. Blood 2001, 97: 1289-1297
23. Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC. Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science 1999, 284: 339-343.
24. Rytomaa M, Lehmann K, Downward J. Matrix detachment induces caspase-dependent cytochrome c release from mitochondria: inhibition by PKB/Akt but not Raf signalling. Oncogene 2000, 19: 4461-4468.

25. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998, 94: 491–501.
26. Puthalakath HA, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DCS, Strasser A. Bmf: A proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science 2001, 293: 1829–1832.
27. Takaoka A, Adachi M, Okuda H, Sato S, Yawata A, Hinoda Y, Takayama S, Reed JC, Imai K. Anti-cell death activity promotes pulmonary metastasis of melanoma cells. Oncogene 1997, 14: 2971–2977.
28. Yawata A, Adachi M, Okuda H, Naishiro Y, Takamura T, Hareyama M, Takayama S, Reed JC, Imai K. Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. Oncogene 1998, 16: 2681–2686.
29. Nishimura S, Adachi M, Ishida T, Matsunaga T, Uchida H, Hamada H, Imai K. Adenovirus-mediated transfection of caspase-8 augments anoikis and inhibits peritoneal dissemination of human gastric carcinoma cells. Cancer Res 2001, 61: 7009–7014.

(Accepted for publication, Jan. 9, 2002)